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An insulin-like growth factor found in hepatopancreas implicates carbohydrate metabolism of the blue crab *Callinectes sapidus*

J. Sook Chung *

University of Maryland Center for Environmental Science, Institute of Marine and Environmental Technology, 701 East Pratt Street, Columbus Center, Baltimore, MD, United States

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ABSTRACT

Hyperglycemia that is caused by the release of crustacean hyperglycemic hormone (CHH) from the sinus gland to hemolymph is one of the hallmark physiological phenomena, occurring in decapod crustaceans experiencing stressful conditions. However, the mechanism(s) by which such elevated glucose levels return to resting levels is still unknown. Interestingly, noted is a difference in the clearance rate of hemolymph glucose between adult females and adult males of the blue crab, *Callinectes sapidus*: the former with more rapid clearance than the latter. The presence of an endogenous-insulin-like molecule is suggested in *C. sapidus* because an injection of bovine insulin, significantly reduces the levels of hemolymph glucose that were previously elevated by emersion stress or the glucose injection. Using 5′ and 3′ RACE, the full-length cDNA of an insulin-like molecule is isolated from the hepatopancreas of an adult female *C. sapidus* and shows the same putative sequence of an insulin-like androgenic gland factor (IAG) but differs in 5′ and 3′ UTR sequences. A knock-down study using five injections of double-stranded RNA of CasIAG-hep (*dsRNA-CasIAG-hep*, 10 μg/injection) over a 10-day period reduces *CasIAG-hep* expression by ~50%. The levels of hemolymph glucose are also kept higher in *dsRNA-CasIAG-hep* injected group than those treated with *dsRNA-green fluorescent protein* (*dsRNA-IAG-hep*) or saline. Most importantly, the hepatopancreas of *dsRNA-CasIAG-hep* injected animals contains amounts of carbohydrate (glucose, trehalose, and glycogen) significantly lower than those of control groups, indicating that the function of CasIAG-hep in carbohydrate metabolism in crustaceans is similar to carbohydrate metabolism in vertebrates.

1. Introduction

The levels of blood/hemolymph sugar, namely glucose, in vertebrates and invertebrates are hormonally regulated. In vertebrates, insulin and glucagon counteract to maintain glucose levels at approximately 5 mM in the blood. In crustaceans, crustacean hyperglycemic hormone (CHH) is responsible for hyperglycemia, elevating levels of hemolymph glucose in response to stress, with this metabolic condition as well noted (Chung et al., 2010; Frajul-Moles, 2006; Webster et al., 2012). However, to date, a mechanism by which such elevated glucose in crustacean hemolymph is returned to basal levels has not been described. More specifically, a hormone that functions as an equivalent to vertebrate and mammalian insulin has not yet been found in crustaceans.

An insulin-like peptide or insulin-like growth factor is known to be phylogenetically ubiquitous, as such molecules are found in organisms across eukaryotes (Piñero-González and González-Pérez, 2011) including mollusks (Floyd et al., 1999; Gricourt et al., 2003, 2006; Lebel et al., 1996; Plisetskaia, 1979, 1978; Sevala et al., 1993) and other invertebrates (Barrett and Loughton, 1987; Duve and Thorpe, 1979; Masumura et al., 2000; Wu and Brown, 2006a) and. Earlier studies report that crustaceans also contain a putative vertebrate insulin-like molecule particularly in the muscle as well as in the hepatopancreas of lobster, *Homarus americanus* (Sanders, 1983a,b). The function of this molecule appears to be associated with carbohydrate metabolism in that the extract of lobster hepatopancreas stimulated glycogen synthesis in mouse/rat tissues (Gallardo et al., 2003). Additionally, the injection of recombinant human insulin increased glycogen content in hepatopancreas of shrimp *Penaeus vannamei* (Gutiérrez et al., 2007). Furthermore, the presence of receptors that are similar to vertebrate insulin receptors have been reported in the hepatopancreas and muscles of two shrimp species *Penaeus monodon* (Lin et al., 1993) and *Penaeus japonicus* (Chuang and Wang, 1994). Overall, these reports indicate that crustaceans may possess insulin or insulin-like peptide, the structure and function of which may be related to a counterpart in mammals and other vertebrates.

* Fax: +1 410 234 8896.
E-mail address: chung@umces.edu

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Recently, a crustacean male hormone (androgenic gland hormone, AGH) has been identified in decapod and isopod crustaceans, and is referred to as an insulin-like androgenic gland factor (IAG) or AGH, due to the structural similarity of this hormone to vertebrate insulin (Chung et al., 2011; Manor et al., 2007; Mareddy et al., 2011; Martin et al., 1998; Ohira et al., 2003; Okuno et al., 1999, 2001). Additionally, the presence of insulin-like molecules is reported in the *Daphnia* genome (Dircksen et al., 2011). While the expression of *IAG* is found only in the androgenic gland of *Cherax quadricarinatus* and *P. monodon* (Manor et al., 2007; Mareddy et al., 2011), the blue crab, *Callinectes sapidus*, shows *IAG* expression in the hepatopancreas of males (CasIAG-hep) (Chung et al., 2011), in addition to the androgenic gland. This finding in a crab species is congruent with an earlier report on the presence of a putative insulin-like peptide in the hepatopancreas of lobster, *H. americanus* (Gallardo et al., 2003).

Vertebrate and mammalian insulin are known for their multiple actions including carbohydrate and lipid metabolism, the uptake of amino acids and increase in cellular permeability of potassium, magnesium, and phosphate ions. Hence, it seems plausible to suggest that a crustacean IAG-like factor may have an additional prototypical function in carbohydrate metabolism. However, the physiological action of IAG, specifically in carbohydrate metabolism, has not yet been examined in any crustacean species.

Herein, this study aimed to define a functional role of the *IAG* that is expressed specifically in the hepatopancreas of the blue crab, *C. sapidus*, mainly on the prototypical metabolic action, similar to the role of vertebrate insulin. The expression profile of *IAG* in various tissues was examined in which female hepatopancreas had expression of *IAG* significantly greater than the male counterpart. With isolation of the full-length *IAG-hep* (*CasIAG-hep*) from the hepatopancreas of an adult female crab, RNAi using double-stranded RNA was carried out to define its function in glucose metabolism.

2. Material and methods

2.1. Animals

Crabs at early juvenile stages (carapace width = CW, 20–30 mm) were obtained from the blue crab hatchery [Institute of Marine and Environmental Technology (IMET), Baltimore, Maryland] and reared to reach adulthood (120–150 mm CW) in artificial sea water at 15–18 ppt in a re-circulated closed system at room temperature (21–22 °C) as described (Katayama and Chung, 2009). Juvenile (70–90 mm CW) and adult crabs only at the intermolt stage (Drach and Tchernigovtzeff, 1967) were used for the experiments.

2.2. Changes in the levels of hemolymph glucose by emersion, glucose injection, and the injection of CasCHH or bovine insulin

Juveniles (70–90 mm CW, n = 6–8/group) were exposed to air for 30 min immediately after the first 100 μl of hemolymph collection. Upon the second hemolymph collection (100 μl) at the end of 30 min emersion, animals received 100 μl of crustacean saline containing 10 pmol of native CasCHH, previously purified from sinus gland extracts as described (Chung and Zmora, 2008). Another group of animals were injected with 100 pmol of bovine insulin (Sigma)/100 ul crustacean saline that was a 10-fold higher dose than CasCHH, because of a heterologous assay. Thereafter, all animals were returned to the water. To ensure delivery of these injection materials into the hemolymph, the crustacean saline was spiked with phenol red at 15 μg/ml crustacean saline. The hemolymph (100 μl) was collected at 60 and 120 min after hormone injection. The control group was treated similarly, but kept in water throughout the experiment.

2.3. The effect of bovine insulin injection on the levels of circulating glucose in adult females and males

In order to further examine if there is a difference in glucose metabolism between adult females and males, 18 mg of glucose/100 μl crustacean saline was injected into adult crabs, immediately after collecting hemolymph samples at t = 0 min. Our preliminary study indicated that two minutes is long enough for the injected glucose to circulate evenly throughout the hemolymph. At t = 2 min, 100 μl of hemolymph was withdrawn from the right hand side of the arthrodial membrane located between the chela and the first walking leg to determine the levels of glucose in hemolymph at t = 2 min. Bovine insulin (160 pmol/100 ul saline) was injected into the same location but on the left-hand side. The hemolymph was collected at 32 and 62 min after the injection of bovine insulin.

2.4. Carbohydrate assays

Glucose levels in hemolymph samples and the glucose, trehalose, and glycogen levels in hepatopancreas tissues were measured by the methods as described (Chung, 2008; Qiong and Chung, 2014).

2.5. 5′ and 3′ rapid amplification of cDNA ends (RACE) of IAG from the hepatopancreas of an adult female C. sapidus

Total RNAs from hepatopancreas samples were extracted using TRIzol® reagent (Invitrogen) and quantified with a NanoDrop spectrophotometer (Thermo Scientific). Cloning of the full-length cDNA of *CasIAG-hep*, 5′ and 3′ RACE cDNAs of hepatopancreas of adult females (total RNA 1–3 μg) were synthesized using a SMART™ cDNA Amplification kit (BD Bioscience) by following the manufacturer's protocol.

A two-step PCR assay for 5′ and 3′ RACE was carried out as described (Chung et al., 2011). In brief, 5′ RACE of *CasIAG-hep* with CasIAG-5R1 primer and 3′ RACE with CasIAG-3F1 primer (Table 1) were used at the initial annealing temperature starting from 57 °C to 50 °C for 8 cycles and 27 cycles at 58 °C. One microliter of the first touch-down PCR reaction was amplified with NUP (BD Biosciences) and CasIAG-5R2 primer for 5′ RACE and CasIAG-3F2 primer (Table 1) for 3′ RACE. Bands were excised for subcloning into a pGEM®-T Easy vector (Promega) and sequencing.

Table 1
Primer sequences for isolating the full-length cDNA of *CasIAG* from the hepatopancreas of female *C. sapidus*, qRT-PCR assay and dsRNA production.

	Primer sequences (5′ to 3′)
CasIAG-3F1	ATCCTTCTCCTCCGTCTGCCTTAC
CasIAG-3F2 (=QF)	CAGATACAATGGGAGTGACTTCCTTTGA
CasIAG-5R1	GTTCACGTATCGGGAGGATCTGC
CasIAG-5R2	CCTTCCTCCTGCCCACTGAGTCTTG
CasIAG-5R3 (=QR)	CTTGAAGAGTTGGAAGGCGTTTTCT
CasIAG-F-dsRNA	TAATACGACTCACTATAGGGATGTGTCTCCGCGTG
CasIAG-R-dsRNA	TAATACGACTCACTATAGGGTCAAAGGAAGTCACT
GFP-F-dsRNA	TAATACGACTCACTATAGGGCCACCGGTCGCCACCA
GFP-R-dsRNA	TAATACGACTCACTATAGGGTTACTTGTACAGCTCG
CasAK-QF	CTACCACAACGACAACAAGACCTTC
CasAF-QR	ACGCGCTTCTCAATCTCGTTA

Forward primers of CasIAG-3F1 and -3F2 were used for 3′ RACE and CasIAG-5R1, 2, and 3 were used for 5′ RACE. CasIAG-3F2 (QF) and -5R3 (QR) primers were used for qRT-PCR analysis. Underlined is the T7 promoter sequence.